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(21) International Application Number: PCT/US93/06589 (22) International Filing Date: 13 July 1993 (13.07.93) (30) Priority data: 07/912,531 13 July 1992 (13.07.92) US (71) Applicant: THE CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 55 Shattuck Street, Boston, MA 02115 (US). (72) Inventor: YANKNER, Bruce, A. ; 299 Prince Street, W. Newton, MA 02165 (US). (74) Agent: FREEMAN, John, W.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).		(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: SCREEN FOR ALZHEIMER'S DISEASE THERAPEUTICS BASED ON β -AMYLOID PRODUCTION (57) Abstract Normal mammalian cells (which generally express APP-encoding DNA) process the APP product so as to provide detectable extracellular levels of β -amyloid peptide. This finding enables an important and long sought screen for compounds that may be suitable therapeutics to treat, prevent, control, or lessen the severity of Alzheimer's disease, as a result of their ability to influence the production of extracellular β -amyloid peptide. The therapeutic capacity of a candidate compound for treating Alzheimer's disease is assessed by: a) providing a population of mammalian cells which expresses amyloid precursor protein (APP), and which produces extracellular β -amyloid peptide; b) culturing that population in a culture medium comprising the candidate compound; and c) measuring the extracellular amount of β -amyloid peptide so as to determine the effect (if any) of the candidate compound on the extracellular amount of β -amyloid peptide.		

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SCREEN FOR ALZHEIMER'S DISEASE
THERAPEUTICS BASED ON β -AMYLOID PRODUCTION

Statement as to Federally Sponsored Research

5 This invention was funded at least in part by a grant from the U.S. Government, and the Government has certain rights in the invention.

Background of the Invention

 This invention relates to screening compositions
10 which may be useful to treat Alzheimer's disease.

 Alzheimer's disease is a common neurodegenerative disorder, characterized by progressive dementia together with the presence of characteristic clinical features. A specific defining group of neuropathological features
15 includes extracellular deposits in the form of amyloid plaques and vascular amyloid, as well as intracellular deposits in the form of neurofibrillary tangles. The plaques and cerebrovascular amyloid contain 6-10nm straight filaments, which are comprised of a 28-43 amino
20 acid subunit, known as the " β -amyloid peptide" or the "A-4 peptide". See, Glenner et al. (1984) *Biochem. Biophys. Res. Commun.* **120**:885-890; Masters et al. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**:4245-4249.

β amyloid peptide is reportedly implicated in the
25 causation of Alzheimer's disease (Goate et al. (1991) *Nature* **353**:844-846) and may be directly responsible for the neuronal death that occurs in Alzheimer's disease

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(Yankner et al. (1989) *Science* 250:279-282). β -amyloid peptide has a high propensity for aggregation and β -pleated sheet structure.

β -amyloid peptide is derived from a larger transmembrane precursor protein (amyloid precursor protein or "APP"), and several different alternatively spliced transcripts of APP have been reported. Kang et al. (1987) *Nature* 325:733-736; Kitaguchi et al. (1988) *Nature* 331:530-532; Ponte et al., (1988) *Nature* 331:525-527; Tanzi et al. (1988) *Nature* 331:528-530; de Sauvage et al (1989) *Science* 245:651-653; Neve and Yankner WO 90/05138; and Neve WO 89/07657. These transcripts have varying sizes, e.g., 695, 751, and 770 amino acid residues. The actual biological function of APP is unresolved, although the protein has been found to increase the survival of cultured cells, is present in regions of cell to cell contact and may play a role in mediating cell substratum adhesion and blood coagulation. (Schubert et al., (1989) *Neuron* 3:689-694; Ueda et al., (1989) *Ann. Neurol.* 25:246-251; Chen et al., (1991) *Neurosci. Lett.* 125:223-226; Van Nostrand et al., (1990) *Science* 248:745-748; Smith et al., (1990) *Science* 248:1126-1128; Shivers et al., (1988) *EMBO J.* 7:1365-2370; Ueda et al., (1990) *J. Neurosci.* 10:3295-3304).

A secreted form of APP is normally generated by proteolytic cleavage of the cellular transmembrane APP (Weidemann et al., (1989) *Cell* 57:115-126). This

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proteolytic cleavage of APP leaves behind in the cell membrane-bound C-terminal fragments of APP which have been demonstrated in the brain and in transfected cells in culture (Selkoe et al., (1988) *Proc. Nat. Acad. Sci. USA* 85:7341-7345; Oltersdorf et al., (1989) *Nature* 341:144-147). Normal proteolytic cleavage of APP occurs within the β amyloid sequence (residue 16 of β amyloid) (Sisodia et al., (1990) *Science* 248:492-495; Esch et al., (1990) *Science* 248:1122-1124), producing a membrane-bound APP derivative containing 16 extracellular amino acids plus the remaining transmembrane and cytoplasmic portions of APP. This process may prevent formation of intact β amyloid peptide. Other cleavages of APP may occur in lysosomes giving rise to larger fragments of APP that may contain β amyloid. Golde et al., *Science* 255:726-730 (1992).

Summary of the Invention

I have established that normal mammalian cells (which generally express APP-encoding DNA) go on to process the APP product so as to provide detectable extracellular levels of β amyloid peptide. This finding enables an important and long sought screen for compounds that may be suitable therapeutics to treat, prevent, control, or lessen the severity of, Alzheimer's disease, as a result of their ability to influence the production of extracellular β amyloid peptide. The invention thus features methods for assessing the therapeutic capacity

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of a candidate compound for treating Alzheimer's disease. The method comprises: a) providing a population of mammalian cells which expresses amyloid precursor protein (APP), and which produces extracellular β amyloid peptide; b) culturing that population in a culture medium comprising the candidate compound; and c) measuring the extracellular amount of β amyloid peptide so as to determine the effect (if any) of the candidate compound on the extracellular amount of β amyloid peptide.

10 In preferred embodiments, the cells are continuous mammalian cell lines including, without limitation, human monkey or rat cells. Also preferably, the cells include exogenous APP-encoding DNA (i.e., DNA artificially introduced into the cells, e. g., by transfection, 15 infection, transformation or other means) effecting expression of APP, particularly overexpression of APP in comparison to the wild type cells without such exogenous DNA. The preferred way to measure the extracellular amount of β amyloid peptide is by combining a sample of 20 culture medium exposed to the cells with an antibody that is specific for β amyloid peptide (or a determinant of it appearing not only in β amyloid peptide but also in β amyloid peptide fragments and in larger β -amyloid-peptide containing proteins such as APP) and then determining 25 antibody- β amyloid peptide binding.

The invention provides a straightforward, fast and relatively inexpensive way to assess therapeutic capacity

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of candidate compounds (particularly large numbers of compounds) *in vitro*.

In a second aspect the invention features methods for assessing the therapeutic capacity of a candidate
5 compound for treating Alzheimer's disease based on the amount of a specific intracellular APP fragment. According to this aspect of the invention, a population of mammalian cells which expresses amyloid precursor protein (APP) is provided and cultured in a culture
10 medium comprising the candidate compound. The intracellular amount of the APP fragment is then measured to determine the effect (if any) of the candidate compound. The APP fragment in question is characterized in that it is approximately 11.5 kd, it includes the 4kd
15 β amyloid peptide sequence, and it extends to the carboxy terminus of APP. Preferably, the cells involved produce extracellular β amyloid peptide.

Other features and advantages of the invention will be apparent from the following description of
20 preferred embodiments and from the claims.

Description of the Preferred Embodiment(s)

Assays according to the invention generally involve culturing cells and exposing them to candidate compounds to assess changes in extracellular levels of β
25 amyloid peptide.

Suitable cell types include neuronal as well as non-neuronal mammalian cells. Apparently, mammalian cells

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generally produce and process APP so as to yield detectable levels of extracellular β amyloid peptide. In particular, any established cell line (particularly primate, and most preferably human, cell lines) can be
5 used. In the experiments described below, COS cells (a continuous cell line derived from monkey kidneys) were used, but those skilled in the field will appreciate that screens according to the invention can be practiced with a large variety of cell lines.

10 In order to improve detectability of extracellular β amyloid peptide, it is useful to engineer the cells to overexpress APP. Overexpression can be accomplished by introducing (e.g., by transfection, transformation, or any other suitable technique) a DNA expression vector
15 into the cell. Such expression vectors will include DNA encoding any of the various forms of APP described above. Such DNA can be obtained by the methods reported above, which are hereby incorporated by reference. The APP-encoding DNA is linked by well known techniques to
20 regulatory DNA that will effect its expression in the chosen host. Those skilled in the art will understand that there are numerous ways to make suitable constructions.

Surprisingly, detection of β amyloid peptide can
25 be accomplished by immunological technological techniques using antibodies that are specific for β amyloid peptide domains, particularly antibodies produced in response to

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a challenge with β amyloid peptide or immunogenic fragments of it. Any of a wide range of well known immunodetection techniques can be used.

In particular, the secreted β amyloid peptide can
5 be detected by radioimmuno techniques (e.g. immunoprecipitation) by adding ^{35}S -methionine to the cell culture, and thereby labeling all proteins. The medium containing the secreted proteins is collected and an antibody to β amyloid is added to recover and quantitate
10 that peptide. For example, beads containing protein G or protein A are added in combination with the anti- β amyloid peptide antibody. The beads are concentrated and collected by centrifugation and then boiled in a buffer containing a detergent (e.g. 1-2% sodium dodecyl sulfate)
15 to release the radioactive protein bound to the beads. The dissociated β amyloid proteins are resolved by polyacrylamide gel electrophoresis to separate them (e.g. a Tricine gel system). X-ray films of the gels indicate labeled β amyloid proteins as bands at
20 approximately 3.5 and 4 kd. The amount of protein in the bands is quantitated by densitometric scanning of the autoradiogram, or by cutting the band out of the gel and measuring radioactivity, or by Western blot.

The antibodies used can be generated to the entire
25 β amyloid peptide or to immunological fragments of it, using standard procedures. Either polyclonal or monoclonal antibodies can be used. Specific fragments

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for raising such antibodies are discussed below, but it is a straightforward matter to determine which β amyloid fragments are suitable by producing numerous candidate fragments and screening the antibodies raised to them for ability to bind the β amyloid peptide.

Specific examples of the extracellular β amyloid peptide detection techniques are described below. Those skilled in the art will understand that the invention is practiced by comparing the results obtained when a candidate compound is included in the culture medium to a control having no candidate compound.

A second aspect of the invention involves detection of an intermediate in the synthesis of β amyloid which I have identified in cells rather than in culture medium. This intermediate is 11.5 kd in size and it contains the 4 kd β amyloid sequence. It extends to the carboxy terminal end of App and can be detected not only by antibodies to β amyloid peptide but also by antibodies to the carboxy terminal domain of APP. Agents which reduce the production of this 11.5 APP fragment from APP also are candidate Alzheimer's therapeutics. To the extent that the examples use cell lysates, they relate to the second aspect of the invention.

The following examples are provided to illustrate (not to limit) the invention.

EXAMPLE 1

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The following experiment describes plasmids used to engineer cells to overexpress APP-encoding DNA. The pCDNA1.APP₆₉₅ and pCDNA1.APP₇₅₁ expressor plasmids express two different versions of full-length APP₆₉₅ (Kang, 1987, 5 cited above) or APP₇₅₁ (Tanzi, 1988, cited above). In these plasmids, cDNAs are under the control of the CMV promoter in the pCDNA.1 vector (Invitrogen Corp.). The 4.2 kb pCDNA1 vector is a derivative of pCDM8 (Seed, (1987) *Nature* 329:840-842) which contains a CMV promoter 10 and enhancer, splice segment, and polyadenylation signal, and SV40 and Polyoma virus eukaryotic origin of replication as well as an M13 origin of replication for the rescue of single-stranded DNA. A pGEM-3 (Promega) plasmid containing a partially digested Eco RI-Eco RI 3.0 15 kb APP₆₉₅ (Kang et al., 1987, cited above) or 3.2 kb APP₇₅₁ (Tanzi et al., 1988, cited above) cDNA insert was digested with Hind III to isolate a 2.9 kb or 3.1 kb Hind III-Hind III fragment containing a full-length APP₆₉₅ or APP₇₅₁ cDNA, respectively, flanked at the 5' end by the 20 pGEM-3 Hind III site and polylinker sequences. The Hind III-Hind III fragments were ligated into the pCDNA1 vector at a unique Hind III site. Single-stranded DNA was made directly from pCDNA1.APP₆₉₅ and pCDNA1.APP₇₅₁ according to the manufacturer's protocol (Invitrogen 25 Corp.). The plasmid DNAs used in transfection experiments were purified by CsCl centrifugation and checked by double stranded DNA sequencing.

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EXAMPLE 2

To produce cells for use in the method of the invention, COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin, and streptomycin. Twenty four hours after seeding, 50% confluent COS-1 cells were transfected with 5 ug of plasmid DNA (produced according to Example 1) per 10 cm² tissue culture dish by the DEAE dextran method (Cullen, (1987) *Methods Enzymol.* 152:684-703).

10

EXAMPLE 3

As an antibody for detecting β -amyloid peptide, it is possible to use rabbit antiserum raised against a peptide containing the C-terminal 20 amino acids of APP, conjugated to an appropriate carrier such as PPD. Other antibodies can be commercially obtained, e.g. the mouse monoclonal antibody Mab22C11 (Boehringer Mannheim), which is reported to be immunoreactive with all forms of APP proteins (Weidemann et al., 1989, cited above). β amyloid antisera can also be raised against other synthetic peptides, e.g. those containing β amyloid peptide residues 1-40 or 28-40. The monoclonal antibody Alz-90 can be raised against a peptide corresponding to amino acids 511-608 of amyloid precursor protein which includes amino acids 1-12 of β amyloid peptide (Boehringer Mannheim). Also, anti-BP16 (Chen, 1991, cited above) can be raised against synthetic peptides containing amino acids 1-16 of β amyloid. For peptide

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preabsorption controls, 3 μ l of antiserum is preincubated with 20 μ g of the peptide to which the antibody was raised overnight at 4°C.

EXAMPLE 4

5 For steady state labeling experiments, COS-1 cells transfected with the APP expressor plasmids were labeled 48 hrs after transfection with 100-150 μ Ci per ml each of [³⁵S] methionine for 16 hours. For pulse-chase experiments, COS cells were labeled 60 hrs after
10 transfection with either [³⁵S] methionine and [³⁵S] cysteine or [³⁵S] methionine alone for 10 minutes, then rinsed twice with PBS, and chased with DMEM supplemented with 10% fetal calf serum and a three-fold excess of unlabeled methionine and cysteine for the indicated time
15 periods.

EXAMPLE 5

To determine the level of labeled β amyloid peptide in the cell supernatants (culture medium exposed to the transfected cells), the culture medium was
20 separated from the cells and cleared of debris by centrifugation at 3000 x g for 20 mins. at 4°C. The Sepharose beads were washed five times and then resuspended in 2X SDS sample buffer and boiled for two SDS sample buffer and boiled for two minutes. The
25 immunoprecipitated β amyloid proteins were analyzed by polyacrylamide gel electrophoresis in 10-20% Tris-

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Tricine-SDS gels and fluorography with En³Hance™ (New England Nuclear).

EXAMPLE 6

To verify the sequence of the proteins at issue,
5 antibody immunoprecipitated protein labeled with [³⁵S]
methionine was resolved by electrophoresis in 10-20%
Tris-Tricine-SDS gels and electroblotted to
polyvinylidene difluoride (PVDF) membranes (Matsudaira, 1987).
The radiolabeled band was identified by autoradiography,
10 excised from the blot, and placed in an ABI Model 475 gas
phase sequencer. The AT3 amino acids were collected at
each cycle into a fraction collector and the
radioactivity determined by liquid scintillation
counting.

15

EXAMPLE 7

The labeled cells were harvested by lysis in a
buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl,
and 5% Triton X-114 at 0°C as previously described
(Gabuzda et al., 1991) in the presence of protease
20 inhibitors (10 µg/ml leupeptin, 100 µg/ml aprotinin, 50
µg/ml antipain, 5 µg/ml pepstatin, 0.3 mM
phenylmethylsulfonyl fluoride) and phosphatase inhibitors
(50 mM imidazole, 50 mM potassium fluoride, 50 mM B-
glycerophosphate, 50 mM sodium pyrophosphate, 100 mM
25 sodium orthovanadate, and 0.1 mM zinc chloride).

Subsequently, the detergent phase containing integral
membrane proteins was isolated by phase separation at

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30°C and centrifugation through a cushion of 6% sucrose, 10mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.06% Triton X-114 by the method of Bordier (Bordier, 1981). The detergent phase was dissolved in RIPA lysis buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) at 0°C and clarified by centrifugation at 13,000 X g for 10 minutes.

EXAMPLE 8

Immunoprecipitated cell lysates were separated by 10-20% Tris Tricine-SDS-PAGE and electrotransferred to nitrocellulose. Nitrocellulose membranes were blocked by 5% nonfat dry milk/0.1% Tween 20 and then incubated with the primary antibody for 12 hours at 4°C. The reaction product was visualized with an alkaline phosphatase-conjugated goat anti-rabbit (1:1000 dilution) (Boehringer Mannheim).

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WHAT IS CLAIMED IS:

1. A method for assessing therapeutic capacity of
a candidate compound for treating Alzheimer's disease,
said method comprising,
providing a population of mammalian cells which
expresses amyloid precursor protein (APP), and which
produces extracellular β amyloid peptide,
culturing said cells in a culture medium
comprising said candidate compound,
measuring the extracellular amount of β amyloid
peptide, and determining any effect of said candidate
compound on the extracellular amount of β amyloid
peptide.
2. The method of claim 1 in which said cells are
monkey, rat or human cells.
3. The method of claim 1 in which said cells
include exogenous DNA effecting expression of APP.
4. The method of claim 1 wherein measuring the
extracellular amount of β amyloid peptide comprises
combining a sample of said culture medium with antibody
that is specific for β amyloid peptide and determining
binding of said antibody to β amyloid peptide in said
medium.

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5. The method of claim 1 in which said cells are non-neuronal cells.

6. The method of claim 1 in which said cells are neuronal cells.

5 7. A method for assessing therapeutic capacity of a candidate compound for treating Alzheimer's disease, said method comprising,

 providing a population of mammalian cells which expresses amyloid precursor protein (APP),

10 culturing said cells in a culture medium comprising said candidate compound,

 measuring the intracellular amount of an APP fragment, said fragment being characterized in that it is approximately 11.5 kd, it includes the 4kd β amyloid
15 peptide sequence, and it extends to the carboxy terminus of APP, and determining any effect of said candidate compound on the intracellular amount of said fragment.

8. The method of claim 7 in which said cells produce extracellular β amyloid peptide.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/06589

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/53; C12Q 1/02; C12P 21/00

US CL :435/7.21, 29, 70.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.21, 29, 41, 69.1, 70.1, 70.3, 948, 172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 57, No. 1, issued 07 April 1989, Weidemann et al, "Identification, Biogenesis, and Localization of Precursors of Alzheimer's Disease A4 Amyloid Protein", pages 115-126, especially see page 115, paragraph bridging first and second columns, page 117, last full paragraph and "Tissue Culture and Transfection" on page 124.	1-6
Y	Neuroscience Letters, Volume 125, issued 1991, Chen et al, "An Antibody to Beta-Amyloid and the Amyloid Precursor Protein Inhibits Cell-substratum Adhesion in Many Mammalian Cell Types", pages 223-226, especially see the Abstract on page 223.	1-6

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

13 October 1993

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Authorized officer

CAROL BIDWELL

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/06589

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A, 90/05138 (NEVE ET AL) 17 May 1990, see entire document, especially see page 7, lines 13-29.	1-8
Y	The Journal of Biological Chemistry, Volume 265, No. 8, issued 15 March 1990, Oltersdorf et al, "The Alzheimer Amyloid Precursor Protein", pages 4492-4497, especially see the Abstract on page 4492, the last sentence of the paragraph bridging the first and second columns on page 4492 and page 4496, second column, first full paragraph.	7-8
X,P	Proceedings of the National Academy of Science USA, Volume 90, issued March 1993, Busciglio et al, "Generation of Beta-Amyloid in the Secretory Pathway in Neuronal and Non-neuronal Cells", pages 2092-2096, especially see the Abstract on page 2092 and page 2094, first column, first full paragraph.	1-8